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Direct PCR sequencing with boronated nucleotides

Kenneth W. Porter, J. David Briley and Barbara Ramsay Shaw*

Department of Chemistry, Duke University, Durham, NC 27708, USA

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ABSTRACT

A method is described to simultaneously amplify and sequence DNA using a new class of nucleotides containing boron. During the polymerase chain reaction, boron-modified nucleotides, i.e. 2′-deoxynucleoside 5′-α-[P-borano]-triphosphates, are incorporated into the product DNA. The boranophosphate linkages are resistant to nucleases and thus the positions of the boranophosphates can be revealed by exonuclease digestion, thereby generating a set of fragments that defines the DNA sequence. The boranophosphate method offers an alternative to current PCR sequencing methods. Single-sided primer extension with dideoxynucleotide chain terminators is avoided, with the consequence that the sequencing fragments are derived directly from the original PCR products. Boranophosphate sequencing is demonstrated with the Pharmacia and the Applied Biosystems 373A automatic sequencers, producing data that is comparable with cycle sequencing.

INTRODUCTION

Current PCR sequencing techniques rely on the incorporation of dideoxynucleotide chain truncators and thus are inherently incompatible with polymerase chain reaction (PCR) amplification (1–8). Once a dideoxynucleotide is incorporated, the chain is terminated and no further extension or amplification is possible. By necessity, amplification must be performed prior to cycle sequencing. An alternative, but relatively unexplored, method for sequencing is to substitute delimiters for truncators directly into the PCR amplification and to reveal the presence of the delimiters either chemically or enzymatically.

Such a sequencing method, based on the incorporation of thiophosphate-modified nucleotides (2′-deoxynucleoside 5′-α-[P-thio]-triphosphates) into PCR products, was proposed by Nakamaye *et al.* (9). The thiophosphates were incorporated into DNA during PCR amplification and their positions were revealed by chemical degradation. An analogous enzymatic approach by Labeit *et al.* (10,11) also used thiophosphates as delimiters to sequence DNA; after incorporation into primer extension products, the positions of the thiophosphates were revealed by exonuclease III digestion. While, in principle, this method could be applied to PCR sequencing, the thiophosphates proved unsatisfactory because of uneven band intensity (9,12).

We have employed boranophosphates (2′-deoxynucleoside 5′-α-[P-borano]-triphosphates) a novel class of nucleic acids (13) synthesized in our laboratory, in order to develop a method that permits both amplification and direct sequencing of PCR products. The boranophosphates are heat stable and can be incorporated into DNA by PCR, yet, once incorporated, they block the action of exonuclease (14,15; K.Porter and B.R.Shaw, unpublished observations). Utilizing the complete set of thymidine-, 2′-deoxyadenosine-, 2′-deoxycytidine- and 2′-deoxyguanosine-5′-α-[P-borano]-triphosphates (dNT^{b}Ps; Fig. 1) in base-specific PCR reactions accomplishes both amplification and base-specific demarcation. The PCR products are then digested with exonuclease to generate the sequencing fragments. As such, PCR sequencing with boranophosphates provides a means for truly direct PCR sequencing.

MATERIALS AND METHODS

The 5′-α-[P-borano]-triphosphates of dA, dT, dC and dG were synthesized and the diastereomers were HPLC separated by modification of methods described previously (14). Oligonucleotide primers were purchased from Genset and purified by TLC. [γ-^{33}P]ATP and [α-^{33}P]dATP (>1000 Ci/mmol) were purchased from Amersham.

Polymerase chain reaction/exonuclease digestion

Phage T7 DNA template (5 ng) was mixed with 20 pmol primers ($T7_{for}$ 5′-GGAGCGTAGGAAATAATAC-3′, positions 34534–34552 of phage T7; $T7_{rev}$, 5′-CGGTTTTAATTACGTTAGCC-3, complementary to positions 35042–35025), 100 μM each of dATP, dTTP, dCTP and dGTP and 2.5 μM of one base-specific boronated dNTP (i.e. dAT^{b}P, dTT^{b}P, dCT^{b}P or dGT^{b}P) in 50 μl Vent buffer [NEB; 10 mM KCl, 20 mM Tris–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100]. An aliquot of 0.5 μl (1 U) Vent DNA polymerase (NEB) was added and PCR was performed for 25 cycles of 95°C for 1 min, 53°C for 1 min and 76°C for 1 min. A 10 μl aliquot of each reaction was digested with 0.5 μl (32.5 U) exonuclease III (Life Technologies) for 30 min at 37°C. Samples were separated on a 1% agarose gel.

Primer extension/exonuclease digestion

Primer (5′-CAGGAACAGCTATGGCCTC-3′; 10 pmol) was 5′-end-labeled with 20 μCi [γ-^{33}P]ATP by polynucleotide kinase. Labeled primer was annealed to unlabeled template (5′-GTGTA-

*To whom correspondence should be addressed. Tel: +1 919 660 1551; Fax: +1 919 660 1618; Email: brs@chem.duke.edu

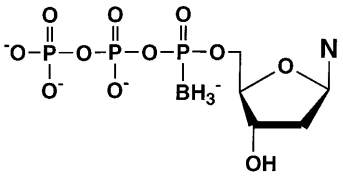

Figure 1. 2′-Deoxynucleoside 5′-α-[P-borano]-triphosphate (dNT^{b}P). N = adenine, cytosine, guanine or thymine.

GCTGAGGCCATAGCTGTTCCTG-3′; 10 pmol). Extension was accomplished with 1 U T4 DNA polymerase in the presence of (i) all-normal dNTPs or (ii) successive replacement of one dNTP with the corresponding dNT^{b}P (50 µM each in 25 µl 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) for 10 min at 37°C. An aliquot of each extended primer–template duplex (10 µl) was digested with 0.5 µl (32.5 U) exonuclease III for 30 min at 37°C. Samples were separated on a 16% polyacrylamide–8 M urea gel.

DNA sequencing

Pharmacia automated sequencer. A sample of 22 ng DNA template (*p53* inserted into pUC18) was mixed with 10 pmol modified $M13_{for}$ (5′-CGACGTTGTAAACGACGGCCAGT-3′; 5′ Cy 5, internal biotin) and 10 pmol unmodified $M13_{rev}$ (5′-ACAGGAAACAGCTATGACCATG-3′; 800 bp product) primers, 25 µM each dATP, dTTP, dCTP and dGTP and either dAT^{b}P (2.5 µM), dTT^{b}P (10 µM), dCT^{b}P (10 µM) or dGT^{b}P (2.5 µM) in 50 µl Taq PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). The reaction mixture was heated to 95°C for 1 min and returned to ice. After addition of 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer) PCR was performed for 25 cycles of 95°C for 15 s, 62°C for 1 min and 76°C for 1 min.

A 25 µl aliquot of the PCR reaction was mixed with streptavidin-linked magnetic beads (Dynal; 10 mg/ml in 25 µl 5 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl) and incubated with mild agitation for 30 min. The PCR products bound to the magnetic beads were immobilized by a magnet and washed twice with exonuclease III buffer (10 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT). The samples were resuspended in 10 µl exonuclease III buffer and digested with 60 U exonuclease III for 30 min at 37°C.

After digestion, the samples were heated for 1 min at 95°C to denature the PCR products. The magnetic beads, along with the biotinylated primer strand fragments, were immobilized with a magnet and the unmodified primer strand fragments were removed. The biotinylated primer strand fragments were resuspended in 10 µl formamide loading buffer, heated for 1 min at 95°C to detach them from the streptavidin-linked magnetic beads and then 6 µl loaded onto a sequencing gel.

As a control, cycle sequencing was performed on *p53*-pUC18 DNA as directed in the Pharmacia AutoCycle sequencing kit. All samples were analyzed on a Pharmacia prototype single color, four lane automatic fluorescent sequencer.

Applied Biosystems 373A sequencer. Dye Primers were provided by PE Applied Biosystems and a human leukemia cDNA library was provided by Stratagene. Base-specific four color PCR reactions were performed with Dye Primers: FAM-labeled primer for dC reactions, JOE for dA, TAMRA for dG and ROX for dT. As suggested for the 373A, two PCR reactions were performed for

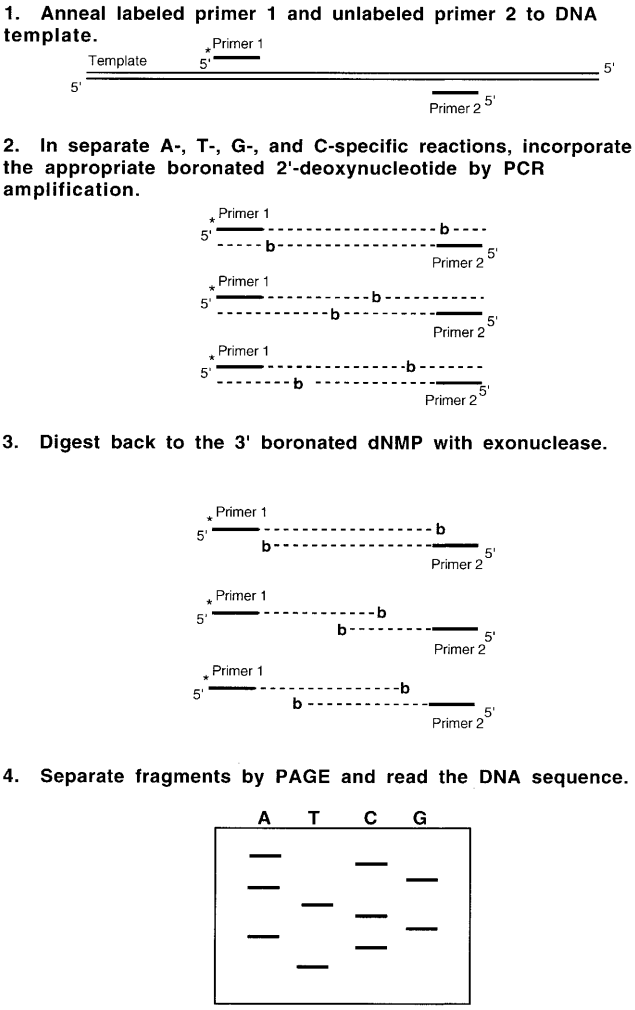

Figure 2. Direct PCR sequencing with boronated nucleotides.

ROX- and TAMRA-labeled samples. A sample of 20 ng DNA template (human cDNA inserted into λZAP II) was mixed with 20 pmol labeled –21M13 (5′-TGTAAACGACGGCCAGT-3′) and 20 pmol unlabeled $M13_{rev}$ (5′-ACAGGAAACAGCTATG-ACCATG-3′; 1000 bp product) primers, dATP, dTTP, dCTP and dGTP (25 µM each), one each of dAT^{b}P (2.5 µM), dTT^{b}P (10 µM), dCT^{b}P (10 µM) or dGT^{b}P (2.5 µM) and 2.5 U Taq DNA polymerase in 50 µl PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). PCR was performed for 30 cycles of 95°C for 15 s, 55°C for 1 min and 70°C for 1 min.

After amplification, the base-specific reactions were combined, extracted with an equal volume of chloroform, precipitated with isopropanol and resuspended in 60 µl water. A 15 µl aliquot was digested with 0.5 U/µl exonuclease III and 1.6×10^{-6} U/µl phosphodiesterase I (from *Bothrops atrox*; Sigma Chemical Co.) in 30 µl exonuclease buffer (10 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT) for 30 min at 37°C. Samples were extracted with chloroform, precipitated with isopropanol and resuspended in 6 µl loading buffer (84% formamide, 4 mM EDTA, 8 mg/ml dextran blue). The samples were heated at 95°C for 2 min and 4 µl were loaded onto a 4.75% polyacrylamide gel and analyzed on an Applied Biosystems 373A Stretch automatic DNA sequencer.

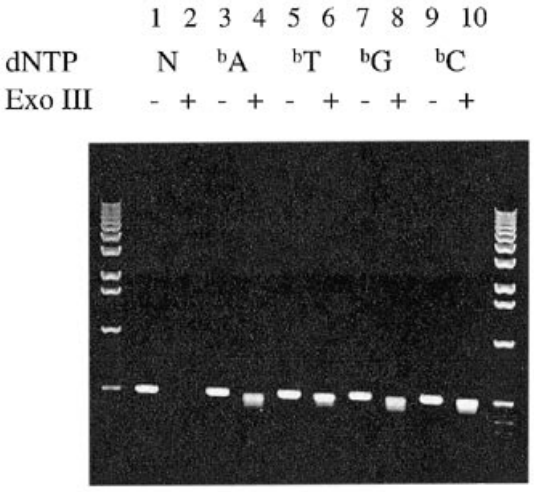

Figure 3. dNT^{b}Ps are substrates for PCR. PCR was performed with all-normal (lane 1) or partially boronated (dAT^{b}P, lane 3; dTT^{b}P, lane 5; dCT^{b}P, lane 7; dGT^{b}P, lane 9) nucleotides. Following amplification, the DNA was treated with exonuclease III: all-normal dNTPs (lane 2) or partially boronated (dAT^{b}P, lane 4; dTT^{b}P, lane 6; dCT^{b}P, lane 8; dGT^{b}P, lane 10) nucleotides.

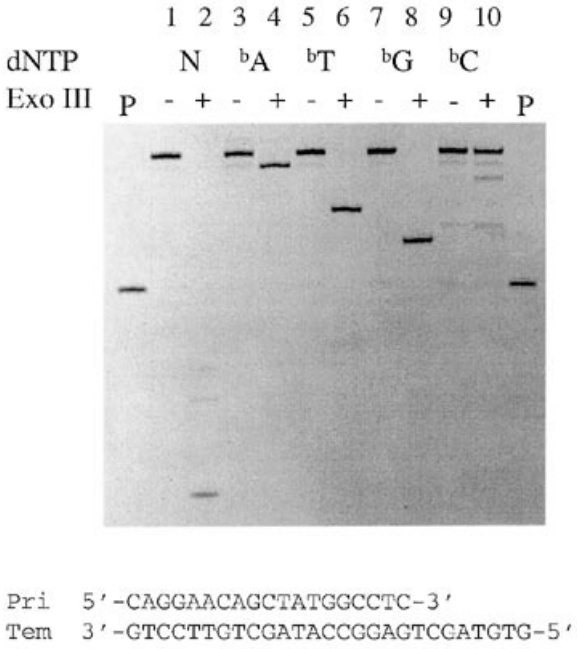

Figure 4. Primer extension with dNT^{b}Ps. Primers were extended in the presence of all normal dNTPs (lane 1) or successive replacement of one dNTP with the corresponding dNT^{b}P (dAT^{b}P, lane 3; dTT^{b}P, lane 5; dCT^{b}P, lane 7; dGT^{b}P, lane 9). Following amplification, the extended primer was treated with exonuclease III: all-normal dNTPs (lane 2) or boronated (dAT^{b}P, lane 4; dTT^{b}P, lane 6; dCT^{b}P, lane 8; dGT^{b}P, lane 10) nucleotides.

Cycle sequencing was performed with the AmpliTaq FS sequencing kit (PE Applied Biosystems) according to the manufacturer's instructions.

RESULTS

Current methods for PCR sequencing cannot, in fact, directly sequence PCR products. DNA templates must first be amplified by PCR and only subsequently sequenced by a secondary primer extension reaction, which incorporates the dideoxynucleotide chain terminators. In contrast, for the boranophosphate sequencing method (Fig. 2), the amplified PCR products, after digestion, are loaded directly onto the sequencing gel.

To accomplish direct PCR sequencing, boranophosphates are incorporated in base-specific PCR reactions and their presence is revealed by exonuclease digestion, thereby defining the sequence of the DNA. In order to evaluate the use of boranophosphates for sequencing, several questions had to be answered. Could the boranophosphates be incorporated into DNA during PCR without decreasing amplification efficiency? Once incorporated, were the boranophosphates sufficiently resistant to exonuclease digestion that the normal nucleotides would be digested leaving a series of borano-terminated DNA fragments? Finally, could the boranophosphate method produce high quality sequence data?

Polymerase chain reaction

To address the compatibility and efficiency of boranophosphates with PCR, a 509 bp duplex was amplified by 25 cycles of PCR in the presence of a small quantity of each of the dNT^{b}Ps. As seen in Figure 3, comparable amounts of full-length DNA were produced with all-normal (lane 1) and partially boronated (lanes 3, 5, 7 and 9) nucleotides, demonstrating that the dNT^{b}Ps are readily incorporated into PCR reactions.

To examine the nuclease resistance conferred by the boranophosphates, the PCR products were treated with exonuclease III, which is a double-strand-specific 3′-exonuclease. The all-normal PCR products were digested extensively (lane 2), while the boron-containing DNA was noticeably resistant to digestion (lanes 4, 6, 8 and 10). Therefore, these studies demonstrated that boronated nucleotides can be incorporated into DNA by PCR and the resultant PCR products are resistant to exonuclease digestion.

Primer extension

To determine whether a boranophosphate could substitute, base specifically, for the corresponding normal dNTP and act as a block to exonuclease, a primer was extended in the presence of 100% of one boronated dNTP (dAT^{b}P, dTT^{b}P, dGT^{b}P or dCT^{b}P) plus the three remaining normal dNTPs (Fig. 4). All of the primers were extended to the full length of the template (lane 1, all-normal; lanes 3, 5, 7 and 9, boronated). The presence of a significant quantity of boronated nucleotide (4–11% of the total nucleotide concentration of the extended primer) did not alter the yield or the electrophoretic mobility. Following extension, an aliquot of each sample was digested with exonuclease III. The all-normal product was digested completely (lane 2), whereas for each boranophosphate-containing sample the exonuclease digestion was halted significantly at the position of the boronated substitution (lanes 4, 6, 8 and 10). It should be noted that the preference of exonuclease III for cytidine residues (16) is also observed for boronated cytidine, thus producing faint extraneous bands in lane 10. Nevertheless, under conditions in which all-normal extension products were digested completely, the boronated extension products were base specifically resistant to exonuclease.

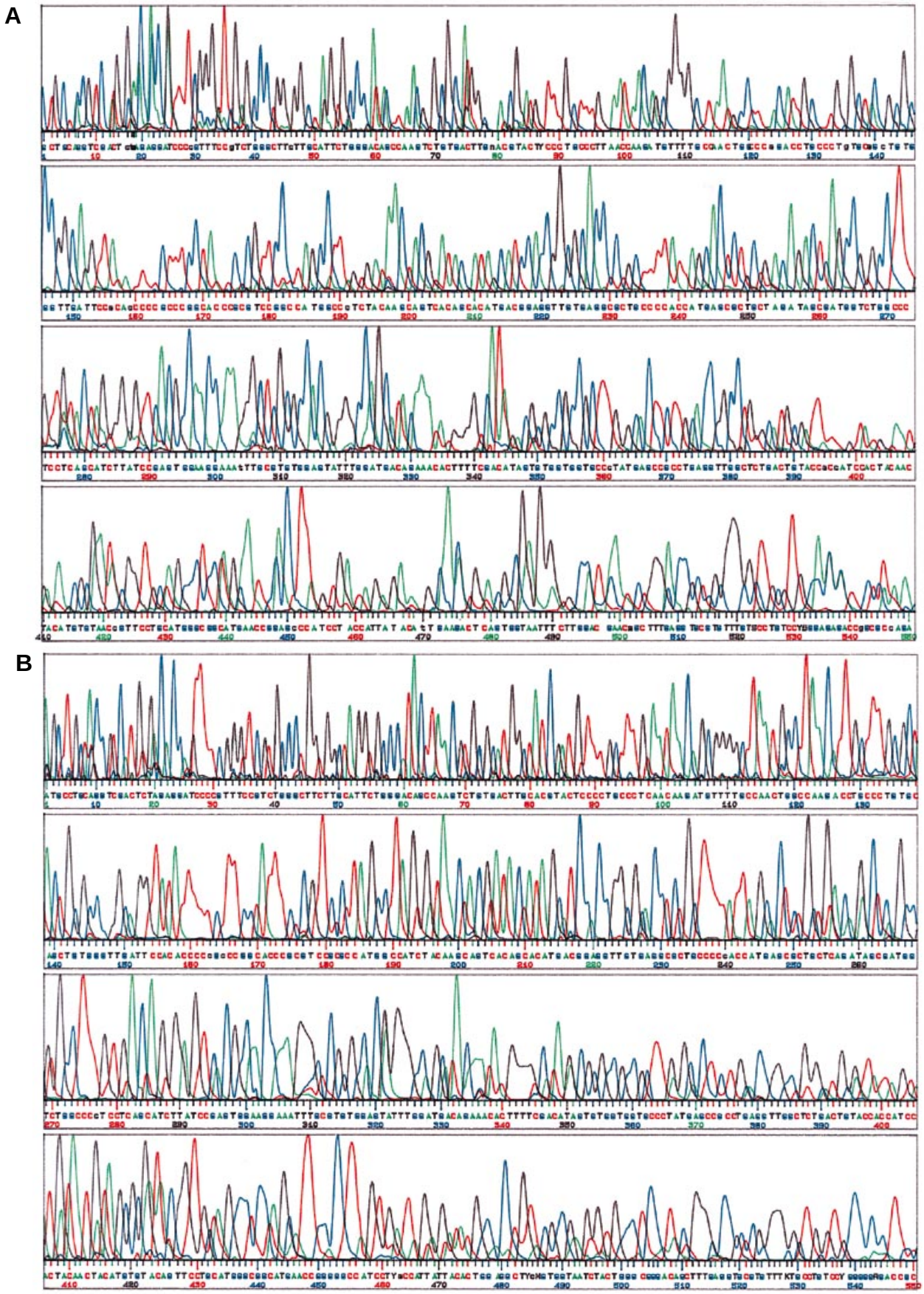

Figure 5. Comparison of boronated PCR Sequencing (**A**) with Taq cycle sequencing (**B**) on the Pharmacia sequencer.

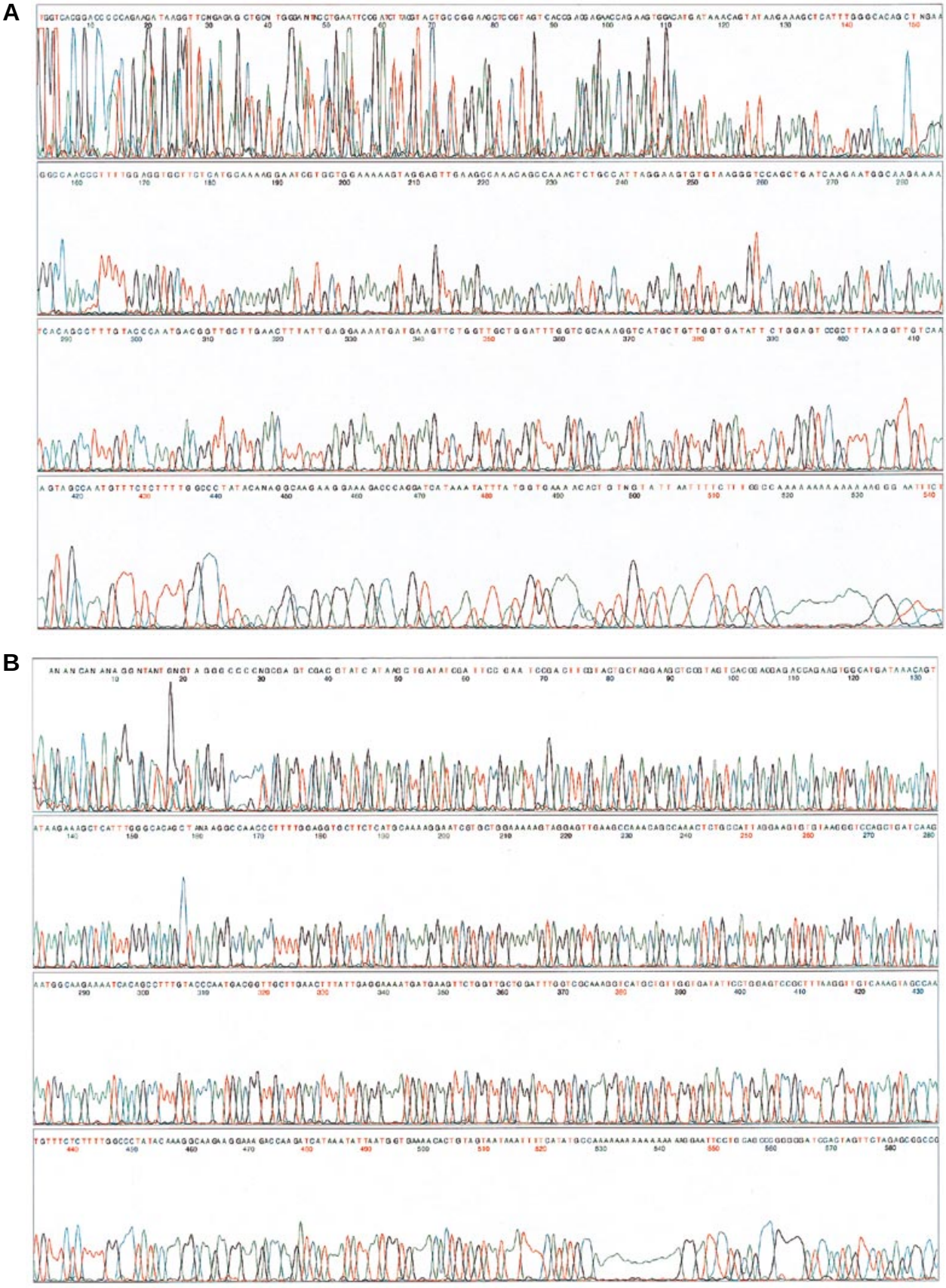

Figure 6. Comparison of boronated PCR Sequencing (**A**) with AmpliTaq FS cycle sequencing (**B**) on the Applied Biosystems 373A.

Taken together, Figures 3 and 4 demonstrate that the boronated nucleotides: (i) do not obstruct PCR or primer extension; (ii) are more resistant to exonuclease III than are normal nucleotides; (iii) are resistant to exonuclease III base specifically. These properties make the boranophosphates suitable for use as the sequence delimiters described in Figure 2.

DNA sequencing

To assess the quality of the sequence data generated by the boranophosphate method, PCR products amplified from double-stranded clones were sequenced (Figs 5 and 6). For comparison, the analogous regions were sequenced by cycle sequencing from purified plasmid templates. Boranophosphate sequencing is compatible with both radioactive (data not shown) and fluorescent labeling. Automated sequencing was accomplished by the single color, four lane format (Pharmacia) and by the four color, single lane format (PE Applied Biosystems).

Pharmacia sequencer. Figure 5 compares boranophosphate and Taq cycle sequencing of the *p53* gene on the Pharmacia instrument. For boranophosphate sequencing, the sequencing primer was modified with both a Cy 5 fluorescent label and biotin, the ligand for streptavidin-linked magnetic beads. Immediately after amplification, the biotinylated PCR duplexes were bound to streptavidin-linked magnetic beads, immobilized with a magnet and the PCR buffer was exchanged for the optimal exonuclease III buffer. Exonuclease III digestion was performed while the PCR products were attached to the magnetic beads, after which the base-specific borano-terminated DNA fragments were removed from the beads and analyzed on the automated sequencer.

Boranophosphate sequencing (Fig. 5A) compares favorably with Taq cycle sequencing (Fig. 5B) methods; for both methods, the sequence could be read over ~450 bases. No secondary structure-induced artifacts were noted for either method, however, each method had at least one position where the correct base could not be determined because of an extremely weak band. At each position where a particular base could not be determined by one method, the base could be called correctly by the other method. The desirability of determining a sequence by two independent methods has been noted previously (17).

Applied Biosystems 373A sequencer. An insert from a human cDNA library was sequenced by direct boranophosphate PCR sequencing and by AmpliTaq FS cycle sequencing on the Applied Biosystems 373A sequencer (Fig. 6). For the boranophosphate method, we performed base-specific PCR reactions with fluorescent Dye Primers, combined the reactions, digested with exonuclease and loaded the fragments onto the sequencer. Digestion conditions were modified to include both exonuclease III and phosphodiesterase I, which improved the signal quality on the Applied Biosystems instrument.

Boranophosphate sequencing (Fig. 6A) approached the quality of AmpliTaq FS cycle sequencing (Fig. 6B) over a considerable range. High quality sequence data were produced for >500 bases by boranophosphate sequencing and for >600 bases by AmpliTaq FS cycle sequencing. Visual inspection of the data showed that the boranophosphate method produced excellent sequence data from positions 100 to 450. Standard ABI base calling software indicated accuracy over an even wider range (Fig. 7); the aligned sequences maintained close agreement from positions 70 to 520.

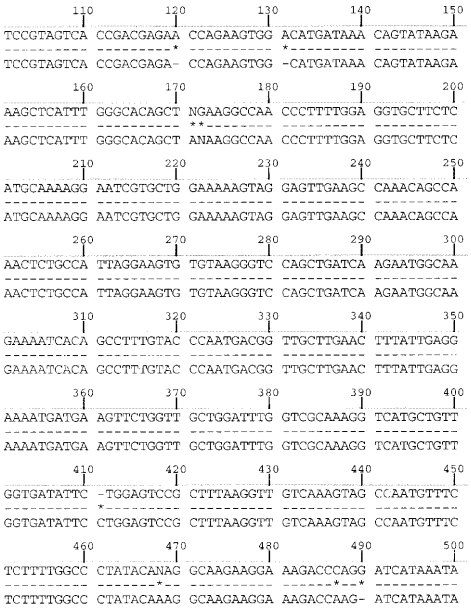

Figure 7. Alignment of sequences derived from boranophosphate PCR sequencing (top) and from AmpliTaq FS cycle sequencing (bottom).

DISCUSSION

We have demonstrated that boranophosphates are suitable for use as delimiters for direct PCR sequencing. The boranophosphates do not inhibit PCR or primer extension and, yet, once incorporated into DNA, they block the action of exonucleases. Boranophosphates, as sequence delimiters, do not interfere with the exponential amplification property of PCR. After incorporation, the positions of the boranophosphates can be revealed by exonuclease digestion, thereby generating a series of borano-terminated fragments that defines the DNA sequence. Since the sequencing fragments are generated from full-length PCR products, the distribution of fragments is advantageously skewed towards the longer fragments.

Direct boranophosphate PCR sequencing has been shown to be compatible with a variety of formats. Manual sequencing has been performed with end-labeled primers, as well as with unlabeled primers and radioactive dNTPs. Automatic sequencing has been performed with two types of sequencers. For boranophosphate sequencing with the Pharmacia instrument, primers were doubly modified with a Cy 5 fluorescent label and with biotin, which allowed solid phase purification of the PCR products. Although not demonstrated here, the boronated PCR products are amenable to bidirectional sequencing either by solid phase strand separation or by uniquely labeled fluorescent primers. For the Applied Biosystems 373A sequencer, a combination of exonuclease III and phosphodiesterase I nucleases was shown to perform optimally. The quality of the resultant sequencing data was equivalent to Taq cycle sequencing and approached that of AmpliTaq FS cycle sequencing.

Presently, boranophosphate sequencing has been shown to generate accurate and reproducible base calls over extended sequences (450 bases in Figs 5–7). Yet in order for boranophosphate sequencing to be more generally useful, the method requires

improvement, i.e. accurate base calls over longer read lengths. We expect to improve our method by introducing modifications to the nucleotides, the exonuclease and the base calling software. Chemically, the structure of the boronated nucleotides can be altered so as to reduce their differential susceptibility to nucleases while maintaining their excellent incorporation properties. Enzymatically, exonuclease III may be modified to perform better in our system, i.e. with reduced sequence specificity, in a manner analogous to that used to design ThermoSequenase (18) and AmpliTaq FS (19,20) polymerases. Finally, the base calling software, including neural net systems (21), can be modified to accommodate any nuances in the data caused by residual differential susceptibility of the boranophosphates to nuclease.

In conclusion, boranophosphate sequencing provides a means for truly direct PCR sequencing. The DNA produced during PCR amplification is transformed by digestion into sequencing fragments and is loaded directly onto the sequencing gel. By virtue of utilizing sequence delimiters, rather than chain terminators, boranophosphate sequencing provides a method that is unique from, and possibly complementary to, cycle sequencing. As such, it should be useful in situations which prove difficult for dideoxy cycle sequencing. Scenarios such as gap filling and closure of large scale sequencing projects, as well as genetic testing and diagnosis, may be appropriate applications for the boranophosphate method.

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REFERENCES

1 Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463.
2 Tabor,S. and Richardson,C.C. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4767.
3 Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horm,G.T., Erlich,H.A. and Arnheim,N. (1985) *Science*, **230**, 1350.
4 Mullis,K.B. and Faloona,F.A. (1987) *Methods Enzymol.*, **155**, 335.
5 Gyllensten,U.B. and Erlich,H.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7652.
6 Innis,M.A., Myambo,K.B., Gelfand,D.H. and Brow,M.A.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9436.
7 Carothers,A.M., Urlaub,G., Mucha,J., Grunberger,D. and Chasin,L.A. (1989) *BioTechniques*, **7**, 494.
8 Sears,L.E., Moran,L.S., Kissinger,C., Creasy,T., Perry-O'Keefe,H., Roskey,M., Sutherland,E. and Slatko,B.E. (1992) *BioTechniques*, **13**, 626.
9 Nakamaye,K.L., Gish,G., Eckstein,F. and Vosberg,H.-P. (1988) *Nucleic Acids Res.*, **16**, 9947.
10 Labeit,S., Lehrach,H. and Goody,R.S. (1986) *DNA*, **5**, 173.
11 Labeit,S., Lehrach,H. and Goody,R.S. (1987) *Methods Enzymol.*, **155**, 166.
12 Olsen,D.B. and Eckstein,F. (1989) *Nucleic Acids Res.*, **17**, 9613.
13 Sood,A., Shaw,B.R. and Spielvogel,B.F. (1990) *J. Am. Chem. Soc.*, **112**, 9000.
14 Tomasz,J., Shaw,B.R., Porter,K., Spielvogel,B.F. and Sood,A. (1992) *Angew. Chem. Int. Edn English*, **31**, 1373.
15 Li,H., Porter.K., Huang,F., Shaw,B.R. (1995) *Nucleic Acids. Res.*, **23**, 4495.
16 Linxweiller,W. and Horz,W (1982) *Nucleic Acids Res.*, **10**, 4845–4859.
17 Koop,B.F., Rowan,L., Chen,W.-Q., Deshpande,P., Lee,H. and Hood,L. (1993) *BioTechniques*, **14**, 442.
18 Tabor,S. and Richardson,C.C. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 6339–6343.
19 Reichert,F.L., Kalman,L.V., Wang,A.M., Gelfand,D.H. and Abramson,R.D. (1995) *Genome Sci. Technol.*, **1**, 46.
20 Spurgeon,S.L., Reichert,F.L., Koeph,S.M., Chen,S.M., Abramson,R.D., Brandis,J., Kalman,L.V. and Wang,A.M. (1995) *Genome Sci. Technol.*, **1**, 48.
21 Golden,J.B., Torgersen,D. and Tibbetts,C. (1993) *ISMB (CCP)*, **1**, 136–144.